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Title: Flow Cytometry Analysis of Immune Cell Subsets within the Murine Spleen, Bone Marrow, Lymph Nodes and Synovial Tissue in an Osteoarthritis Model

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Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **Y**

If **Yes**, can you record movies/images using your own microscope camera?

N

If **No**, JoVE will need to record the microscope images using our scope kit (through a camera port or one of the oculars). Please list the make and model of your microscope.

Leica M80 and we have a non-working Leica DFC295 camera attached to it.

2. Software: Does the part of your protocol being filmed demonstrate software usage? **N**

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Patrick Haubruck**: Our protocol enables the detailed identification and analysis of monocytes, macrophages, and T cells and their relevant subsets within the murine synovium and various other tissues of the immune system **[1]**.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

REQUIRED:

1.2. **Patrick Haubruck**: Our technique facilitates the reliable harvest of live immune cells from the synovium and other relevant tissues for a comprehensive characterization of the osteoarthritic immune response **[1]**.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

Ethics Title Card

1.3. Procedures involving animal subjects have been approved by the Institutional Animal Care and Use Committee (IACUC) and the Northern Sydney Local Health District.

Protocol

2. Spleen, Contralateral Bone Marrow, Ipsilateral Lymph Nodes, and Synovial Tissue Isolation

2.1. For the isolation of tissues of interest from an osteoarthritis model mouse, with the mouse in a supine position, wipe the chest, abdomen, and legs with 70% ethanol **[1]**.

2.1.1. WIDE: Talent wiping mouse, with ethanol container visible in frame *Videographer: More Talent than mouse in shot* **TEXT: Euthanasia: cervical dislocation**

2.2. Use scissors to carefully open the skin along the midline of the abdomen while leaving the abdominal cavity intact **[1]** and gently pull the skin on the right side of the animal away from the underlying muscle, leaving the subcutaneous adipose tissue attached to the skin **[2-TXT]**.

2.2.1. Incision being made

2.2.2. Skin being pulled **TEXT: Use scissors to cut through sporadic adherences as necessary**

2.3. Place the mouse under a dissecting microscope **[1]** and use two curved fine forceps to gently tease out the adipose tissue located at the thigh **[2]**.

2.3.1. Talent placing mouse under microscope *Videographer: More Talent than mouse in shot*

2.3.2. SCOPE: Tissue being teased

2.4. After identifying the three crossed vessels, use fine dissecting forceps to remove the inguinal lymph node located at the intersection of the vessels, taking care not to rupture the capsule **[1]**.

2.4.1. SCOPE: Shot of vessels and LN, then LN being removed *Videographer: Important step*

2.5. Use the forceps to remove the remaining fat from the surface of the lymph node **[1]** and open the abdominal cavity. If no remaining fat can be seen, place the lymph node immediately in a previously prepared and labelled 6-well plate filled with 0.5ml of RPMI media **[2]**. NOTE: Authors added some extra VO sentences but no extra shots, so it will have to be spread out over existing ones, even though at times it doesn't seem like it will match.

2.5.1. SCOPE: Fat being removed
2.5.2. SCOPE: Incision being made

2.6. Use fine scissors to excise the spleen **[1]** and to gently extract the intestines to expose the aorta and its bifurcation. Hereafter, place the spleen immediately in a previously prepared and labelled 6-well plate filled with 0.5 milliliters of RPMI media **[2]**.

2.6.1. SCOPE: Spleen being excised
2.6.2. SCOPE: Intestines being pulled out while being left intact

2.7. Remove the iliac lymph node located at the terminal segment of the abdominal aorta and the origin of the common iliac artery **[1]** and carefully remove the skin from both hind limbs. Immediately place the lymph node in a previously prepared and labelled 6-well plate filled with 0.5 milliliters of RPMI media, pooling all lymph nodes from two animals **[2]**.

2.7.1. SCOPE: Shot of LN, then LN being harvested
2.7.2. SCOPE: Skin being pulled at top of limb OR skin being removed right at bottom of limb

2.8. Using fine scissors or a scalpel, clean the left femur of the attached muscle tissue **[1]** and carefully disconnect both the stifle and the hip joint, leaving the whole bone intact while removing the femur. Then, place the femur in a previously prepared and labelled 6-well plate filled with 0.5 milliliters of RPMI media **[2]**.

2.8.1. SCOPE: Muscle being removed
2.8.2. SCOPE: Hip being disconnected

2.9. Identify the patella tendon of the right stifle joint **[1]** and use fine scissors to remove the adjoining muscle tissue proximal to joint until approximately 5 millimeters of quadriceps tendon proximal to the patella are exposed **[2]**.

2.9.1. SCOPE: Shot of tendon *Videographer: Important step; Video Editor: please emphasize tendon is appropriate/necessary*
2.9.2. SCOPE: Muscle being removed/quadriceps being exposed *Videographer: Important step*

2.10. Cut through the quadriceps tendon approximately 3-4 millimeters proximal to the patella to form a handle **[1]** and use fine forceps to gently pull the tendon away from the joint to expose the edges of the joint capsule attachment **[2]**.

2.10.1. SCOPE: Tendon being cut *Videographer: Important step*

2.10.2. SCOPE: Tenon being pulled/edges being exposed *Videographer: Important step*

2.11. Starting at the femur and moving toward the tibia, use a scalpel to carefully cut along the edges of the joint capsule on both sides while maintaining a gentle traction on the quadriceps tendon **[1]**.

2.11.1. SCOPE: Scalpel cutting along capsule *Videographer: Important/difficult step*

2.12. When the synovial tissue block is attached only to the tibia, the intraarticular fat pad should be clearly visible distal to the patella **[1]** and can be gently detached from the joint and anterior aspect of the menisci **[2]**.

2.12.1. SCOPE: Shot of fat pad *Videographer: Important/difficult step; Video Editor: please emphasize fat pad if necessary/appropriate*
2.12.2. SCOPE: Fat pad being detached *Videographer: Important/difficult step*

2.13. Then cut along the remaining part of the joint capsule to remove the synovial tissue block and immediately place it in a previously prepared and labelled 6-well plate filled with 1.5 milliliters of RPMI media **[1]**.

2.13.1. SCOPE: Scalpel cutting along capsule/tissue being removed *Videographer: Important step*

3. Tissue Processing: Spleen

3.1. When all of the tissues have been collected, place two pooled spleens per condition onto a 70-micrometer cell strainer in a 15-milliliter tube **[1]** and use a sterile, 3-milliliter syringe plunger to gently macerate the tissues through the mesh filter **[2]**, flushing the strainer frequently with a total of 6-milliliters of RPMI 1640 (R-P-M-I sixteen-forty) medium supplemented with 10% FBS (F-B-S) **[3-TXT]**.

3.1.1. WIDE: Talent adding spleens to filter
3.1.2. Spleens being mashed **TEXT: RPMI: Roswell Park Memorial Institute; FBS: fetal bovine serum**

3.2. Sediment the cells by centrifugation **[1-TXT]** and resuspend the pellet in 5 milliliters of red blood cell lysis buffer **[2]**.

3.2.1. Talent placing tube(s) into centrifuge **TEXT: 5 min, 500 x g, RT**
3.2.2. Shot of red pellet, then buffer being added to tube, with buffer container visible in frame

3.3. After 5 minutes, stop the reaction with 10 milliliters of PBS **[1]** and centrifuge the cells

[2-TXT].

3.3.1. Talent adding PBS to tube, with PBS container visible in frame
3.3.2. Talent placing tube(s) into centrifuge **TEXT: Repeat lysis until no visible RBC**

3.4. When no more red blood cells are observed, resuspend the pellet in 1 milliliter of fresh PBS for counting **[1]**.

3.4.1. Shot of white pellet, then PBS being added to tube, with PBS container visible in frame

4. Tissue Processing: Lymph Node and Bone Marrow

4.1. For lymph node processing, place all four lymph nodes onto a 70-micrometer cell strainer in a new 15-milliliter tube **[1]** and use a new, sterile 3-milliliter plunger to gently tease the lymph nodes through the mesh into a single cell suspension. Then, centrifuge the cells at 500 x g for 5 minutes, discard the supernatant, and resuspend the pellet in 500 microliters of PBS for counting **[2-TXT]**.

4.1.1. WIDE: Talent adding LN to strainer
LN being mashed
4.1.2. Filter being flushed, with medium container visible in frame **TEXT: Flush strainer with 6 mL medium to collect**

4.2. To isolate the bone marrow, use a tissue thumb forceps to carefully grasp the intact femur **[1]** and use sharp scissors to cut off the very end of the proximal femur **[2]**.

4.2.1. Femur being grasped
4.2.2. Femur being cut

4.3. Insert a 23-gauge needle into the middle of the intercondylar notch of the femur **[1]** and rotate the needle while applying gentle pressure to drill a hole into the notch **[2]**.

4.3.1. Needle being inserted
4.3.2. Hole being drilled

4.4. Changing the needle as necessary, flush the bone contents of two femurs with 6 milliliters of RPMI supplemented with 10% FBS onto a 70-micrometer cell on a new 15-milliliter tube **[1-TXT]** and use a new 3-milliliter syringe plunger to gently press the bone marrow through the mesh. Hereafter, perform the RBC step as demonstrated for the spleen. When no more red blood cells are observed, resuspend the pellet in 1 milliliter of fresh PBS for counting **[2-TXT]**. NOTE to VO: Long one, maybe split into 2 parts

4.4.1. Bone being flushed onto filter **TEXT: Bone should appear white after complete flushing**
4.4.2. Bone marrow being pressed through filter **TEXT: Rinse strainer with 3 mL medium**
4.4.3. Talent rinsing strainer

5. Tissue Processing: Synovial Tissue

5.1. For processing of the synovial tissue, use fine surgical scissors to dice the two synovial tissue blocks into tiny pieces **[1]** and transfer the samples into a new 15-milliliter tube **[2]**.

5.1.1. WIDE: Talent dicing blocks
5.1.2. Talent adding samples to tube

5.2. Rinse the collection container with 500 microliters of medium to collect any remaining cells and synovial tissues **[1]** and pool the wash in the sample collection tube **[2]**.

5.2.1. Well being rinsed, with medium container visible in frame
5.2.2. Talent adding wash to tube

5.3. Next, add a sufficient volume of enzyme to achieve in a 1 unit/milliliter final concentration **[1]** and digest the synovial tissue sample at 37 degrees Celsius for 2 hours of a rotator. Ensure sufficient movement when placing the tubes in the rotator **[2]**.

5.3.1. Talent adding enzyme to tube, with enzyme container visible in frame
5.3.2. Talent placing tube onto rotator

5.4. At the end of the incubation, stop the digestion with 8 milliliters of RPMI supplemented with 10% FBS **[1]** and filter the cell suspension through a 70-micrometer cell strainer into a new 15-milliliter tube. Rinse the old 15-milliliter tube with 5 milliliters of medium and use this to wash the filter **[2-TXT]**.

5.4.1. Talent adding medium to tube, with medium container visible in frame
5.4.2. Talent adding cells to strainer **TEXT: Wash filter with 5 mL medium**

5.5. Then sediment the cells by centrifugation **[1-TXT]** and resuspend the pellet in 500 microliters of PBS for counting **[2]**.

5.5.1. Talent adding tube to centrifuge **TEXT: 10 min, 500 x g, RT**
5.5.2. Shot of pellet if visible, then PBS being added to tube, with PBS container visible in frame

6. Monocyte and T cell Subset Panel Immunostaining

6.1. To perform viability staining of the isolated immune cells, add 5 x 10^5 cells of each cell suspension to the appropriate wells of two different 96-well, U-bottom plates **[1]** and collect the cells to the bottom of each well. Always include sufficient unstained cells of each tissue type as negative controls **[2-TXT]**.

6.1.1. WIDE: Talent adding cells to well(s), with both plates visible in frame
6.1.2. Talent placing plate into centrifuge *Videographer/Video Editor: shot will be used again* **TEXT: 5 min, 500 x g, 4 °C**

6.2. Wash cells with 200 microliters of PBS **[1]** and resuspend the pellets in 100 microliters of cell-impermeant amine-reactive dye per well for a 15-minute incubation at 4 degrees Celsius protected from light **[2-TXT]**.

6.2.1. Talent adding PBS to well(s), with PBS container visible in frame
6.2.2. Dye being added to well(s), with dye container visible in frame **TEXT: See text all solution preparation details**

6.3. At the end of the incubation, wash the cells two times in 200 microliters of FACS (facks) buffer per wash **[1-TXT]** and resuspend each pellet in 100 microliters of the appropriate antibody of interest or control cocktail. If both intra- and extracellular staining is conducted in the same panel, perform the extracellular staining step now **[2]**.

6.3.1. Talent adding buffer to well(s), with buffer container visible in frame **TEXT: FACS: fluorescence-activated cell sorting**
6.3.2. Talent adding antibody to well(s), with antibody containers visible in frame

6.4. After a 30-minute incubation at 4 degrees Celsius protected from light, wash the cells two times with 200 microliters of FACS buffer per well **[1]** and resuspend the cells in the monocyte subset panel plate in 250 microliters of FACS buffer supplemented with 1 millimolar EDTA. Conduct this step only if no intracellular staining is planned, otherwise move on to the intracellular staining aspect of the protocol **[2]**.

6.4.1. Use 6.1.2. Talent adding plate to centrifuge
6.4.2. Buffer being added to tube, with buffer + EDTA container visible in frame

6.5. Then transfer each monocyte subset sample into the appropriate corresponding FACS tube on ice protected from light **[1]**.

6.5.1. Talent adding sample to tube on ice, with both plates visible in frame

6.6. For intracellular staining of the T cell subset panel cells, resuspend the pellets in 200

microliters of fixation buffer from an appropriate fixation and permeabilization kit for a 40-minute incubation at 4 degrees Celsius protected from light **[1]**.

6.6.1. Talent adding buffer to well(s), with buffer container visible in frame

6.7. At the end of the incubation, wash the cells two times with 200 microliters of permeabilization wash buffer **[1-TXT]** and resuspend the pellets in 100 microliters of the appropriate antibody of interest or control cocktail for a 40-minute incubation at 4 degrees Celsius protected from light **[2]**.

6.7.1. Use 6.1.2. Talent placing plate into centrifuge **TEXT: 5 min, 750 x g, 4 °C, x2**
6.7.2. Talent adding antibody to well(s), with antibody container(s) visible in frame

6.8. Then wash the cells two times in 200 microliters of permeabilization wash buffer per wash **[1]** and resuspend the cells in 250 microliters of FACS plus EDTA buffer **[2]** before transferring the samples into the appropriate corresponding FACS tubes on ice protected from light **[3]**.

6.8.1. Use 6.1.2. Talent adding plate to centrifuge
6.8.2. Buffer being added to tube, with buffer + EDTA container visible in frame
6.8.3. Talent adding sample to tube on ice, with both plates visible in frame

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?

2.4., 2.9.-2.13.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?

2.11., 2.12.

Results

7. Results: Representative Mouse Osteoarthritis Model Immune Cell Subset Analysis

7.1. Here the hierarchical gating strategy for the monocyte subset panel on immune cells gathered from the bone marrow of DMM (D-M-M)-treated animals can be observed **[1-TXT]**.

7.1.1. LAB MEDIA: Figure 1 **TEXT: DMM: destabilization of the medial meniscus**

7.2. Unstained controls can be used to determine the true negatives for the dead-alive stain **[1]** and the gates can be adjusted each time the experiment is conducted as necessary **[2]**.

7.2.1. LAB MEDIA: Figure 2A *Video Editor: please emphasize gate in Control plot*
7.2.2. LAB MEDIA: Figure 2A *Video Editor: please emphasize gates in Sham and DMM plots*

7.3. In this representative analysis, immune cells were isolated from the synovial tissues and stained with extracellular surface markers 6 weeks after DMM or sham-control surgery **[1]**.

7.3.1. LAB MEDIA: Figure 3

7.4. A higher percentage of Ly-6C (lie-six-C)-positive, MHC (M-H-C)-two-negative **[1-TXT]** and M2 macrophages are observed in DMM-treated animals **[2]** compared to the cell populations observed in sham surgery mice **[3]**.

7.4.1. LAB MEDIA: Figure 3 *Video Editor: please emphasize cells in DMM G7 gate*
7.4.2. LAB MEDIA: Figure 3 *Video Editor: please emphasize cells in top right quadrant of DMM CD206 vs CD80 plot* **TEXT: MHC: major histocompatibility complex**
7.4.3. LAB MEDIA: Figure 3 *Video Editor: please emphasize cells in Sham G7 gate and cells in top right quadrant of Sham CD206 vs CD80 plot*

7.5. Here the hierarchical gating strategy for the extra- and intracellular T-cell panel on immune cells isolated from the spleen of DMM-treated animals can be observed **[1]**.

7.5.1. LAB MEDIA: Figure 4

7.6. A higher percentage of Th1 (T-H-one) cells are observed in the lymph nodes **[1-TXT]** and synovial fluid of DMM animals **[2]**, as well as higher numbers of T-regulatory cells **[3]** and Th17 cells **[4]**.

7.6.1. LAB MEDIA: Figures 5 and 6 *Video Editor: please emphasize cells in G9 gate in Figure 5 DMM plot* **TEXT: Th: T helper**

7.6.2. LAB MEDIA: Figures 5 and 6 *Video Editor: please emphasize cells in G9 gate in Figure 6 DMM plot*

7.6.3. LAB MEDIA: Figures 5 and 6 *Video Editor: please emphasize cells in top right/G11 quadrant of DMM plots*

7.6.4. LAB MEDIA: Figures 5 and 6 *Video Editor: please emphasize cells in top right/G12 quadrant of DMM plots*

Conclusion

8. Conclusion Interview Statements

8.1. **Patrick Haubruck**: To ensure the acquisition of high-quality samples, the tissues must be harvested in a swift, meticulous, and consistent manner **[1]**.

8.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (2.4., 2.7., 2.13.)

8.2. **Patrick Haubruck**: Once the immune cells have been isolated, other downstream analyses can be conducted, such as RT-PCR, cell culture, and Western blot, to shine a light on molecular processes of interest **[1]**.

8.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera